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Identification and cloning of a sequence homologue of dopamine β -hydroxylase

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Abstract

We have identified and cloned a cDNA encoding a new member of the monooxygenase family of enzymes. This novel enzyme, which we call MOX (monooxygenase X; unknown substrate) is a clear sequence homologue of the enzyme dopamine β -hydroxylase (DBH). MOX maintains many of the structural features of DBH, as evidenced by the retention of most of the disulfide linkages and all of the peptidyl ligands to the active site copper atoms. Unlike DBH, MOX lacks a signal peptide sequence and therefore is unlikely to be a secreted molecule. The steady-state mRNA levels of MOX are highest in the kidney, lung, and adrenal gland, indicating that the tissue distribution of MOX is broader than that of DBH. Antisera raised to a fusion protein of MOX identifies a single band of the expected mobility by Western blot analysis. MOX mRNA levels are elevated in some fibroblast cell strains at replicative senescence, through this regulation is not apparent in all primary cell strains. The gene for MOX resides on the q arm of chromosome 6 and the corresponding mouse homolog has been identified. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: monooxygenase; cDNA; senescence; chromosomal localization

1. Introduction

Dopamine β -hydroxylase (DBH) catalyses a key step in catecholamine biosynthesis; the conversion of dopamine to norepinephrine (Kaufman and Goldstein, 1965) and is widely conserved amongst mammals. The enzyme is expressed in noradrenergic and adrenergic neurons of the central nervous system, as well as the chromaffin cells of the adrenal medulla (Axelrod, 1972). DBH mRNA levels are affected by growth factors (Hwang and Choi, 1995), cyclic AMP (Lewis et al., 1987;

Stachowiak et al., 1990a) and calcium (Stachowiak et al., 1990b). Since DBH catalyses the conversion of a molecule that lowers blood pressure (dopamine) into one that elevates blood pressure (noradrenaline), inhibition of this enzyme has been targeted as a means of treating hypertension (Kruse et al., 1986; Li et al., 1996). In humans, genetic DBH deficiency results in profound orthostatic hypotension (Robertson et al., 1991), and in mice, targeted disruption of DBH results in embryonic lethality (Thomas et al., 1995). Interestingly, DBH-deficient embryos can be rescued by adrenergic agonists, or a synthetic precursor of noradrenaline, but upon reaching adulthood, these mice display impaired maternal behaviour (Thomas and Palmiter, 1997). Thus, DBH activity plays an important role in embryonic development and in adult behaviour.

DBH shares homology with the monooxygenase domain of the bi-functional enzyme peptidyl-glycine amidating monooxygenase (PAM) (Eipper et al., 1992). Both enzymes catalyze two-electron monooxygenation reactions that are ascorbate-dependent and involve the stereospecific replacement of a methylene hydrogen by hydroxyl (Klinman, 1996). The recent description of

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Abbreviations: cAMP, adenosine 5'-3'-cyclomonophosphate; DBH, dopamine β -hydroxylase; EST, expressed sequence tag; GST, glutathione S-transferase; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; MOX, monooxygenase X; PAGE, polyacrylamide gel electrophoresis; PAM, peptidylglycine α -amidating monooxygenase; PDL, population doubling level; RT-PCR, reverse transcriptase-polymerase chain reaction; UTR, untranslated region.

the crystal structure of the monooxygenase domain of PAM clearly shows two subdomains, each containing a copper atom bound by peptidyl side-chain ligands (Prigge et al., 1997). DBH and PAM have distinct substrate preferences (dopamine for DBH, peptidylglycine for PAM), but both enzymes display activity to a rather broad range of substrates (Klinman, 1996). Co-crystallization of the monooxygenase domain of PAM with a synthetic substrate reveals that the substrate is bound primarily through hydrogen bonds at the active site, while the peptide side chains are held by non-specific contacts, thus providing a rationale for the broad permissiveness of the enzyme with regards to substrate (Prigge et al., 1997).

We have investigated the alteration of gene expression patterns that accompanies replicative senescence in normal human cell strains using a modified version of the differential display technique and have catalogued many genetags that are dysregulated at senescence (Linskens et al., 1995). One of several cDNAs identified by this study is reported here and encodes a novel homolog of DBH.

2. Materials and methods

2.1. cDNA isolation and analysis

Genetags whose expression levels were altered in senescent human fibroblasts (Linskens et al., 1995) were used as hybridization probes to identify homologous clones from a cDNA library prepared from BJ human foreskin fibroblast poly A⁺ mRNA (Lark Technologies, Houston, TX). The cDNA inserts of these clones were isolated by restriction digestion, sonicated to an average size of 500 bp and subcloned into pBluescript II (Stratagene, La Jolla, CA, USA). DNA sequence analysis was obtained using an ABI 377 automated fluorescence DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and the results were compiled using the GeneWorks and ABI Prism software packages.

Analysis of the sequences showed that one cDNA, GC6, corresponding to the genetag GS6 (Linskens et al., 1995), included an extended open reading frame (ORF) that showed amino acid similarity to dopamine β -hydroxylase (DBH). The ORF extended from the 5'-end of clone GC6, indicating that the cDNA was not full-length. Subsequently, a probe fragment was generated using the PCR that corresponded to the 5'-most sequence of the cDNA. Screening of a λ GT11 cDNA library produced from HEK 293 cell mRNA with this probe identified a second clone (λ 3-1-4). DNA sequence analysis confirmed that λ 3-1-4 extended the existing ORF and included a likely initiator Met codon. The

consensus cDNA sequence has been deposited in the American Type Culture Collection (ATCC).

2.2. Cell strains

Normal diploid human cell strains were grown in humidified incubators at 37°C at 5% CO₂. BJ fibroblasts were provided by Jerry Shaw (University of Texas, Southwestern Medical Center at Dallas). C4 and MA fibroblasts strains were established from human skin samples and human umbilical vein endothelial cells (HUVECs) were obtained from the ATCC. Cells were passaged serially and population doubling levels (PDL) were determined by split ratios. For BJ fibroblasts, early passage cells were defined as PDL 25–35, late passage cells were PDL 90–93. For C4 fibroblasts, early passage cells were defined as PDL 20–30, late passage cells were PDL 48–50. For MA fibroblasts, early passage cells were defined as PDL 10–15, late passage cells were PDL 48–50. For HUVECs, early passage cells were defined as PDL 15–25, late passage cells were PDL 50–52.

2.3. Northern blot

A total RNA blot from human tissues was obtained (Northern Territory, Invitrogen, San Diego, CA, USA) and probed with a random-hexamer primed, ³²P-labeled MOX cDNA fragment (nucleotides 1514–1839) according to the manufacturer's instructions. The blot was exposed to a Phosphor Imager screen (Molecular Dynamics, Sunnyvale, CA) for 2 days.

2.4. RT-PCR

Total RNA from cell lines was prepared as described (Linskens et al., 1995). Total RNA samples from tissues were obtained from ClonTech (Palo Alto, CA, USA). cDNA was prepared from total RNA using random hexamer primers and SuperScript II reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD, USA) as described (Feng et al., 1995). PCR was performed using cDNA and GC6-specific primers in the presence of ³²P- α -dATP. Parallel reactions were performed with varying amounts of input cDNA to ensure the amplifications were within the linear range. Reaction products were resolved using polyacrylamide gel electrophoresis (PAGE) and then exposed to phosphorimager screens (Molecular Dynamics). Similar reactions were performed using 28S ribosomal RNA-specific primers. Signal from the appropriate bands were quantified and the MOX signal was then normalized using the signal from the corresponding 28S reaction. For all RT-PCR analyses, results are reported as the average of at least three signals within the linear range of amplification.

2.5. Fusion protein and antisera production

A *EcoRI* restriction fragment (including nucleotides 286–1168; Fig. 1) was prepared from plasmid GC6 and sub-cloned into the *EcoRI* site of the GST-fusion vector pGEX-5X-3 (Pharmacia, Piscataway, NJ, USA). The resulting plasmid, pGEX-GC6, was introduced into *E. coli* strain DH5 α . Bacterial cultures were then grown at 37°C in LB medium containing 100 μ g/ml ampicillin to an $A_{600\text{nm}}$ of approximately 0.5 and fusion protein (GST–MOX) expression was induced by the addition of IPTG to a final concentration of 1 mM. The cultures were continued for 6 h and the bacteria were collected by centrifugation. The bacterial pellet was resuspended in cold PBS and then sonicated briefly. Triton X-100 was added to a final concentration of 1% and the suspension was shaken gently for 30 min. The suspension was centrifuged briefly and the supernatant was removed. The pellet, enriched for inclusion bodies, was resuspended in PBS containing 1% sarkosyl, gently mixed for 30 min, and centrifuged briefly (Burgess, 1996). The supernatant was collected and then denatured by boiling in the presence of β -mercaptoethanol. The sample was loaded onto preparative SDS–PAGE gels (10% acrylamide; BioRad) and resolved at 30 mA constant current. The fusion protein band was visualized by briefly staining the gel with 0.1% Coomassie stain in water, excised and then destained in water. The gel fragment was macerated prior to injection into host rabbits (Babco, Richmond, CA). Antisera was harvested after a secondary antigen boost.

Attempts to bind the GST–MOX fusion protein to GS-agarose were unsuccessful, thus precluding affinity purification of the antisera. Instead, a column matrix was prepared to remove non-specific antibody activities from the serum as follows. Cultures of bacteria hosting the parental plasmid pGEX-5X-3 were prepared as above and the 1% Triton X-100 supernatant was recovered. This material was added to Affigel 15 matrix (BioRad, Hercules, CA) and coupled overnight at 4°. The coupled matrix was washed extensively with PBS containing 1% Triton X-100 and then packed as a column. Crude antisera was then passed over the column and the flow-through re-applied. The column was resolved with PBS and eluted fractions were assayed for reactive antisera by Western blot analysis of extracts from bacteria expressing the pGEX-GC6 vector. For Western blot analysis, gel-purified GST–MOX was first exchanged into factor Xa protease buffer (100 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1 mM CaCl₂) and cleaved according to the supplier's instructions (New England Biolabs, Beverly, MA, USA).

Protein extracts of whole cells were prepared and 20 μ g of soluble protein were loaded onto SDS–PAGE gels, resolved and transferred onto Nytran membranes

TTTCC	AAGGAAGGAAAGGCACACAA	TGSTATCAAAATGTCATTCA	45
TTCCATCTCTTGATGCTCTA	CGATATATATCAGTCTACACT	ATGCTTTTCTGAAAGGCCAG	105
AAGTTCAAAGATGGACTAGT	TTCCAGGGACCTGATTCC	CAGTTGGAATATCCAGCCC	165
CTTGGAAATTCCTCCGGATTT	<u>ATTGAATAACTCTAGACAAC</u>	AAGACTTTTGCTTTTAAAGT	225
M N S F L S	V F R Y P D F	S F P Y F P	
CCATGAATTCCTTTCTCTC	TGTAITTTAGTATCTCTGATT	TTTCTTTCCATATTTTCCA	285
Q D Y F T N A	N R E L K K D	A Q Q D Y H	
CAGGATTTATTTTACAAATGC	AAATAGACAGTTTGAAAGAG	ATGCTCAGCAGATTTACCAT	345
L E Y A M E N	S T H T I I E	F T R E L H	
CTAGAATATGCCATGGAAAA	TAGCACACACACAATAATTG	AATTTACACAGAGCTGCAT	405
T C D I N D K	S I T D S T V	R V I W A Y	
ACATGTGACATAAATGACAA	GAGTATAACGGATAGCAGT	TGAGAGTGATCTGGGCCCTAC	465
H H E D A G E	A G P K Y H D	S N R G T K	
CACCATGAAGATGCAGGAGA	AGCTGGTCCCAAGTACCATG	ACTCCAATAGGGGCACCAAG	525
S L R L L N P	E K T S V L S	T A L P Y F	
AGTTTCCGGTTATTTGAATCC	TGAGAAACCTAGTGTGCTAT	CTACAGCCTTACCATACTTT	585
D L V N Q D V	P I P N K D T	T Y W C Q M	
GATCTGGTAATCAGGACGT	CCCATGCCCAACCAAGAGT	CAACTGATTTGGTGCACAAATG	645
F K I P V F Q	E K H H V I K	V E P V I Q	
TTTAAGATTCCTGTGTTCOA	AGAAAGCATCATGTATTAQA	AGGTTGAGCCAGTGTATACG	705
R G H E S L V	H H I L I L P	C S N N F N	
AGAGCCCATGAGATCTGGT	GCACACATCTCTGTATTC	AGTGCAGCAACACTTTAAC	765
D S V L E S G	H E C Y H P N	M P D A F L	
GACAGGTTCTGGAGTCCGG	CCACGAGTGTATCACCCCA	ACATGCCCGATGCATTCTC	825
T C E T V I F	A W A I G G E	G F S Y P P	
ACTCTGGAACTCTGATTTT	TGCTGGGCTATTTGGTGGAG	AGGGCTTTTCTTATCCACCT	885
H V G L S L G	T P L D P H Y	V L L E V H	
CATGTGGATTTATCCCTTGG	CATCTCATTTAGATCCGCAAT	ATGTGCTCTAGAAATTCAT	945
Y D N P T Y E	E G L I D N S	G T C L R F Y	
TATGATTAATCCACTTATGA	GGAGGCTTATATAGATTAAT	CTGGAGTCTGATTTATTTAC	1005
T M D I R K Y	D A G V I E A	G L W V S L	
ACAATGATATTAAGGAAATA	TGATGCTGGGGTGTATGAG	CTGGCCTCTGGGTGAGCCTC	1065
F H T I P P G	M P E F Q S E	G H C T L E	
TTCCATACCATCTCCCTCAGG	GATGCCGTGAGTTCAGCTCTG	AGGGTCACTGCACCTTTGGAG	1125
C L E E A L E	A E K P S G I	H V F A V L	
TGCCCTGGAAGAGGCTCTGGA	AGCCGAAAGCCAGGTGGAA	TTTCACTGTGTCTGTCTTCT	1185
L H A H L A G	R G I R L R H	F R K G K E	
CTCCATGCTCACTCTGGCTGG	CAGAGGCATCAGGCTGGCTC	ATTTTGGAAAAGGGAAGGAA	1245
M K L L A Y D	D D F D F N F	Q E F Q Y L	
ATGAATTAAGTCTGCTTATGA	TGATGATTTTGTCTTCAATC	TCCAGAGTGTTCAGTATCTA	1305
K E E Q T I L	P G D N L I T	E C R Y N T	
AAGGAACCAACAATCTTT	ACCAGAGATAACCTTAATTA	CTGATGTCTGCTACACACG	1365
K D R A E M T	W G G L S T R	S E M C L S	
AAAGATGAGCTCAGATGAC	TTGGGGAGGACTAAGCACCA	GGAGTGAATGTGTCTCTCA	1425
Y L L Y Y P R	I N L T R C A	S I P D I M	
TACCTTCTTTATTAACCAAG	AATTAATCTTACTCTGATGTG	CAAGTATTTCCAGACATTATG	1485
E Q L Q F I G	V K E I Y R P	V T T W P F	
GAACAACCTTCACTTCAITGG	GGTTAAGGAGATCTACAGAC	CAGTACACAGCTTGGCCTTTC	1545
I I K S L K Q	Y K N L S F M	D A M N K F	
ATATACAAAGCTCTCAAGCA	ATATAAAACCTCTTCTTTCA	TGGATGCTATGAATAAGTTT	1605
K W T K E G	L S F N K L V	L S L P V N	
AAATGACTAAAGGAAGG	TCTCTCTTCAACAGCTGG	TCTCTAGCCTGCCAGTGAAT	1665
V R C S K T D	N A E W S I Q	G M T A L P	
GTCAGATGTTCACAGACAGA	CAATGCTGAGTGTGTGATTC	AAGGATGACAGCATTTACCT	1725
P D I E R P Y	K A E P L V C	G T S S S S	
CCAGATATAGAAAGACCTTA	TAAAGCAGAACCTTTGGTGT	GTCGACGCTCTCTTCTCTCT	1785
S L H R D F S	I N L L V C L	L L L S C T	
TCCCTGCACAGATTTCTC	CATCAACTTGTCTGTGTGCC	TTCTGCTACTCAGCTGCACG	1845
L S T K S L *			
CTGAGCACCAGAGCTTGTG	ATCAAAATCTGTGTGGACTT	GACAATGTTTCTATGATCT	1905
GAACTGTCTATTTGAAGTAC	AGGTAAAGACTGTGTCCAC	TTTGGGCATGAAGGTGTGG	1965
AGACTTTTCTTCCCAATTTT	CCCTCCCTCTCTTTTCTCTTT	CCATGTTTACATGAGAGACAT	2025
CAATCAGGTTCTCTCTCTTT	TTCTAGAAATATCTGATGTT	ATATATACATGGTCAATATA	2085
<u>ATTAAGTGGCTTCACTTAA</u>	<u>GATTAACCATTTTAAAAAAT</u>	<u>GGGCTGTCACTGTGGGATTA</u>	2145
<u>ATGAATTTCTTTTCTCTAC</u>	<u>TACATTTCTGTTTATTTTAA</u>	<u>TACTCATTTGTCTATTTTCA</u>	2205
CTTTTGTGACTTGTCTTTAT	ATTCTTTTAAAAAATTTCTTT	CTTTTAAAAAATATAAAAA	2265
GGACTACGTCTTCTTCCAGT	TTTCTCTCTCTTGTGTGTTCT	TTCTAGTGCTGATTTTCAAG	2325
TGTAACAGGCATTTCTTCTG	ACTTTTAAATTTGTCCAGTTC	TGGTCTTTTCTGTGAATTAC	2385
CAGTGGGCCCCCTTACCTCAA	TGCTTTTGTGTGATGCCAC	TCTGGTTCCTTGTGTTATCT	2445
GAGTCTGTGTGGTACCCAAA	TGACCCACACCCATTTTAA	AGTACTTTTCTTCACTTCTC	2505
CTGTTTTGTACTGCCCAGAT	GAGTTTCTTCTAGAGCTCTG	TCACATCTGAAAGGAAGA	2565
GGCTATGGGAACATAGAAA	TGGTATGTATTAATACTGA	TCATAGGCTGAGGAGAAAA	2625
ATGTAGCTGGCTGCAAAACC	AGTGTGTGTGAGGTGACTTGA	ATGAGGTTCCAGATCAAGA	2685
CAGGCGGTGTGAGCCAGTCC	AGGAGGGTGTAGTTCTGAA	TGGTCTCTTGTGACTTTGG	2745
GTGACACATGTACCACATAC	TGGCTCAGTTTAAAGTCATGG	TTCTATTTGTAGATTTATTTT	2805
TATATATAGTTAATAATGAC	TTTAAATTTGTCAACAATTTGA	AAATCTTGTCACTCTTTTGTG	2865
TTTCTTTTATATAGCTCAGC	CAAATCTCTGTTTATGTTCC	TGCTCTCATCTCTTAAAGCTA	2925
AATCTGTTTGGATCATATTA	<u>ATAAACTCGTGGCG</u>		2960

Fig. 1. cDNA sequence of MOX mRNA. DNA sequence analysis was performed on overlapping MOX cDNA clones, providing a conceptual translation of the protein. An in-frame stop codon in the 5'-non-coding sequence is boxed and potential polyadenylation signal sequences in the 3'-untranslated region are underlined.

A

DBH	MREAAFMYST AVAIFLVILV AALQGSAPRE SPLPYHIPLD PEGSLELSWN	50
MOX		
DBH	VSYTQEAHF QLLVRLKAG VLFMSDRGE LENADLVWLW TGGDTAYFAD	100
MOX	DYFTNANREL KKDAQQDYHL EYAMENSTHT IIEFTIRELHT CDINDKSTHD	70
DBH	A-WSDQKGQI HLDQQDYQL LQVQRTPEGL TLLFKRPFGT CDPKDYLLID	149
MOX	STVRVIWAYH HED-AGEAGP KYHDSNRGK SLRLINPE-K TSVLSTALPY	118
DBH	GTVHLVYGIL EEPFRSLEAI NSGSLQMGLO RVQLLKNIP EPELPSDACT	199
MOX	FDLVNQDVPI ENKLTITYWQ MFKTEVFQEK HHVIRMEFVI QRGHESLVHH	168
DBH	MEVQAPNIQI ESQHTITYWCY IKELFKGFSR HHILKMEHIV TKGNEALVHH	249
PAM	LDIRMPGV-T EKQSDITYFCM SMRLTEV-DEE AFVLDFFE-- -RASMDTVHH	103
MOX	ILLYQCSNNF NLSVLESGHE CYHPNMPDAF LITETMIFAW AIGGEGFSYP	218
DBH	MEVQCAPEM D-SVPHFSGP CDSKMKPDEL NYORHMLAAW ALGAKAFMYP	298
PAM	MLLFQ--NM PSSTGSYWF- CDEGTCTLK- ---ANILYAW ARNAPPTRIE	146
MOX	PHVGLSLGHE LDPHYVILEV HYIN-PTYEE GLINDSGIRI FYTMDIRKYD	267
DBH	EEAGLAFQGE GSSRYLRLEV HYIN-ELVIE GRNDSSGIRI YYTAKLRFN	347
PAM	KGVGFVVGGE TGSRYFVLDV HYGDISAFRD NNKDCSGVSI HLIRLPQPLI	196
MOX	AGVTEAGLVV SLFHTIPFGM PEFQSEGGCT LEQLEEALEA EKPSGIHIFA	317
DBH	AGIMELGLVY TPVMAIPERE TAEILTQYCT DKQTQIAL-- -FESGIHIFA	394
PAM	AGMY---LMM SVDTVLHAGE KVNNSDISCH YKNYPM-----FIFA	233
MOX	VILLHAFLAGR GIRLRHRRKG KEMKLLAYED DFDNFQEFQ YLKEEQITILP	367
DBH	SCLFTHLTGR KVVTVLVRDG REWEIVNQIN HYSPPHOFIR MLKKVVSVEP	444
PAM	YRVEFTHLCK VVSGYRVENG QWTLIGRQSP QLPQAFYPVG HPVDVSF---	280
MOX	GINLTIECFY NTKDRAEMTW GGLSTRSEMC LSVLLIY--- -PRINLTIFA	413
DBH	GIVLTISCTY NTEDELATIV GGFGLSEMC VNVVHY--- -EOTQLELCK	490
PAM	GITLAARCMF TGEGRTEATH TGGTSSLEMC NLYIMYMEA KHAVSFMTCT	330
MOX	SIFTIMEQLQ FIGVKEIYRP VTTWPFIIKS LKQYKNLSFM DAMNKFKWK	463
DBH	TAVLAGFLQK YFHLINRFNN EDVCTCPQAS VSC----- -QFTSVFVNS	532
MOX	KEGLSFNKLIV LSIPVNVFCS KTDNAEWSIQ GMTALFEDIE RPYKAEPLVC	513
DBH	FNRDVLKALY SFAPISMHCN KSSAVRFQGE WNLQPLKVI STLEPTPOC	582
MOX	GTSSSSSLHR DFSINLLVCL LLLSCTLSTKS L	545
DBH	HTSQG----E SPAGPTVSI -----GGQKG-	603

Fig. 2. (A) Amino acid sequence comparison of human MOX (this study), DBH (translated from GenBank deposit X13255) and the monooxygenase catalytic core of PAM (translated from Genbank deposit M37721). Sequences were aligned manually and residues shared between MOX and DBH or MOX, DBH and PAM are boxed. ■, Cys residues conserved in MOX, DBH and PAM; □ Cys residues conserved only in MOX and DBH; ● Predicted peptidyl copper ligands. (B) Disulfide bridging patterns for MOX, DBH, and PAM. The disulfide bridging pattern for the human DBH homodimer was inferred from the pattern assigned for bovine DBH (Robertson et al., 1994), and the human PAM pattern was inferred from that of rat PAM (Kolhekar et al., 1997). The pattern for MOX is proposed based on the conservation of Cys residues between DBH and MOX. (C) Kyte–Doolittle plot comparison of MOX and DBH. Plots were generated using the GeneWorks 2.5 software package (Intelligenetics, Mountain View, CA) and were then aligned as in (A).

(Schleicher and Schuell). Blots were blocked in the TBS buffer containing 0.1% Tween 20 and 5 mg/ml of non-fat powdered milk. Purified anti-MOX antisera was applied at a 1:500 dilution in the block solution. Following serial washes in TBS buffer containing 0.1%

Tween 20, the secondary antibody (alkaline phosphatase-conjugated goat-anti rabbit IgG, Gibco/BRL, Gaithersburg, MD, USA) was applied at a 1:1000 dilution in block solution. NBT/BCIP (Gibco/BRL) was used to develop the blot.

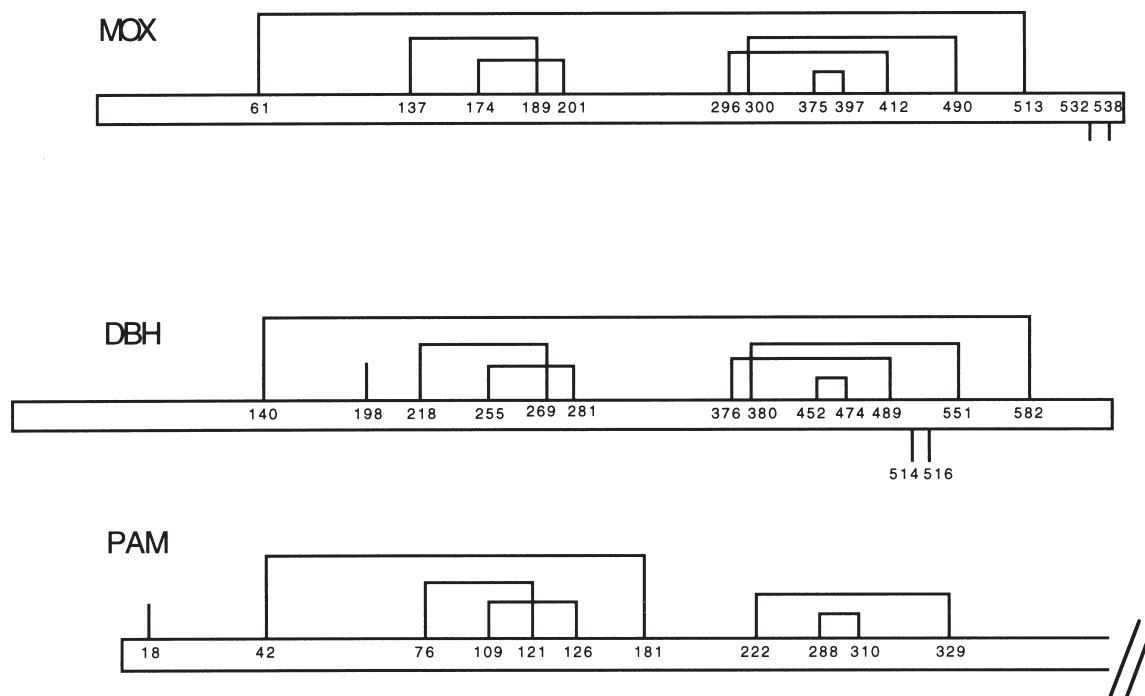
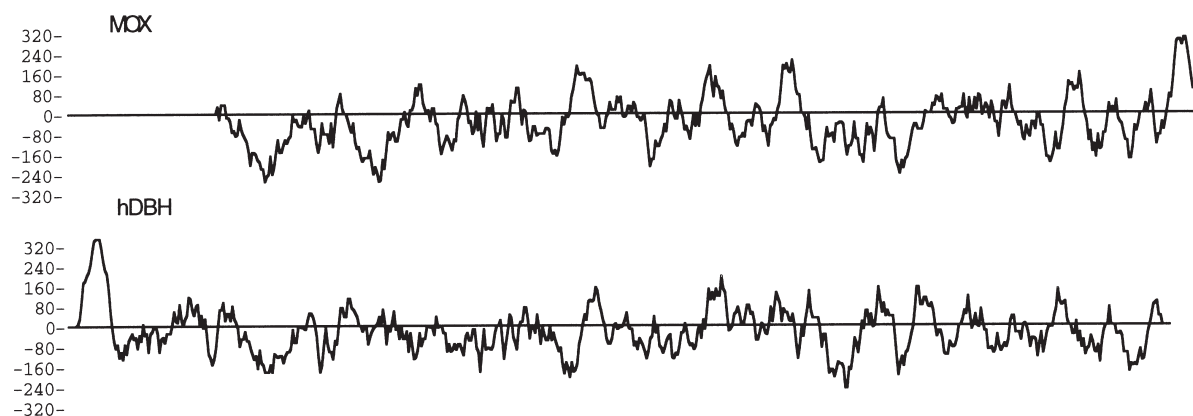
B**C**

Fig. 2. (continued).

2.6. Radiation hybrid panel mapping

GC6-specific primers were used for PCR with genomic DNA from the Stanford G3 RH Panel (Research Genetics, Huntsville, AL). The PCR products were resolved on agarose gels and scored for identity with the same reaction performed on pMOX plasmid DNA. The results were tabulated and processed through the radiation hybrid server at the Stanford Human Genome Center (rhserver@shgc.stanford.edu).

3. Results

3.1. cDNA cloning and DNA sequence analysis

Our initial differential display catalog of senescence-dysregulated genes from human diploid fibroblasts contained both known and novel genetag sequences (Linskens et al., 1995). We isolated one clone, GC6, that corresponded to the original genetag GS6. GC6 encoded an extensive open reading frame that shared

significant homology with dopamine β -hydroxylase (DBH), an enzyme that converts dopamine to norepinephrine (Lamouroux et al., 1987). We have called the predicted protein MOX (monooxygenase X: unknown substrate). The isolation of an additional clone, λ 3-1-4, generated a longer cDNA encoding an extensive open reading frame (ORF) (Fig. 1). The cDNA contains a likely initiator Met, based on the occurrence of an upstream, in-frame, stop codon. In vitro transcription–translation of this cDNA provides a protein whose mobility is consistent with this assignment (data not shown). The sequence surrounding this codon appears to be a relatively weak match to a derived initiator consensus sequence (MOX: AGGTCCTATGA Consensus: GCCAGCCATGG; Kozak, 1987). To verify the sequence in this region, we performed RT-PCR on total RNA from BJ fibroblasts using MOX-specific primers that flank the proposed initiator codon. DNA sequence analysis of the resulting PCR products confirmed the length of the open reading frame and the presence of upstream stop codons. Within the 3' UTR of the message, multiple consensus polyadenylation signals are apparent (Fig. 1). The overall DNA sequence identity between human DBH and MOX is low (25%). Since these proteins share obvious homology, the evolutionary rooting of these two genes must be deep. A BLAST search of the mouse EST database identified corresponding sequences for mouse MOX (ESTs AA388297, AA068006, AA199428), and a contig of these ESTs shares 84% DNA sequence identity and 88% amino acid sequence identity with human MOX (spanning nucleotides 784–1289 of the human MOX mRNA) (data not shown).

3.2. Amino acid sequence comparisons

The predicted amino acid sequence of MOX is clearly homologous to human DBH, as shown in Fig. 2. The DBH monomer is characterized by structural features that include the presence of multiple disulfide bonds that have been assigned by peptide analysis of the bovine enzyme (Robertson et al., 1994). Most of these Cys residues are conserved in MOX (Fig. 2B), though two Cys residues proposed to form intermolecular bonds in DBH (Cys 514 and 516 in the human enzyme) are absent in MOX, and two non-homologous Cys residues are found at the C-terminus of MOX. Further biochemical characterization of the MOX protein will be required to determine if these C-terminal Cys residues form a novel disulfide bond, or form intermolecular bonds, as is the case with DBH. A further subset of Cys residues is conserved across the catalytic core of MOX, DBH and PAM, suggesting that the overall architecture of these cores is similar. Over this region, MOX and DBH share approximately 40% amino acid identity, while MOX and PAM share 23% identity.

The recent description of the crystal structure of the monooxygenase domain of peptidylglycine α -hydroxylating monooxygenase (PAM) (Prigge et al., 1997) has confirmed many of the predicted structural features of this enzyme, such as peptidyl copper ligands and the placement of residues that contribute to catalytic activity (Kolhekar et al., 1997; Boswell et al., 1996; Eipper et al., 1992; Yonekura et al., 1996). MOX maintains all of the peptidyl copper ligands seen in PAM, as does DBH (Fig. 2A). Thus, MOX is predicted to display monooxygenase activity. A co-crystal structure of PAM with substrate also identified several residues that interacted directly with substrate. Only one of these substrate interacting residues (PAM Y318), is conserved in DBH (Y477) and MOX (Y400). In PAM, this residue anchors the terminal carboxyl of the substrate at the active site and mutations in this residue increase K_m but do not affect V_{max} , a result consistent with a role in substrate binding (Kolhekar et al., 1997). In DBH, the native substrate (dopamine) lacks a terminal carboxyl, though one might predict in this case that the residue serves to anchor the terminal amine.

The most striking difference noted between the primary sequences of DBH and MOX is the lack of a signal peptide sequence at the N-terminus of MOX (Fig. 2C) and thus MOX is unlikely to be secreted. The signal sequence of DBH has been investigated extensively and both cleaved and uncleaved versions of the DBH molecule have been identified, as have soluble and membrane-bound forms of the enzyme (Winkler et al., 1970; Dhawan et al., 1987; Lewis and Asnani, 1992). The 5'-untranslated region of the MOX mRNA may have a cryptic signal peptide sequence that has been interrupted by a stop codon, suggesting that MOX may have originated from a gene that, like DBH, encoded a signal peptide.

3.3. mRNA expression patterns

Northern blot analysis of total RNA from BJ fibroblasts indicates a major MOX transcript of approximately 3.3 kb (Fig. 3A). An examination of the 3'-non-coding region of the MOX cDNA reveals multiple polyadenylation sites (AATAAA) (Fig. 1), however, we cannot at present determine with precision the preferred sequence. The relative expression of MOX transcripts appeared highest in lung, kidney and brain. RT-PCR analysis confirmed high levels in the kidney and lung and also suggested modest expression in the testes (Fig. 3B). To extend this analysis, a dot blot of total RNA (ClonTech, Palo Alto, CA, USA) from a wide variety of human tissues was probed; the results confirmed high levels of MOX expression in lung and kidney and also showed high levels of expression in fetal kidney, uterus and adrenal gland tissues (data not shown). Protein analysis has consistently demonstrated

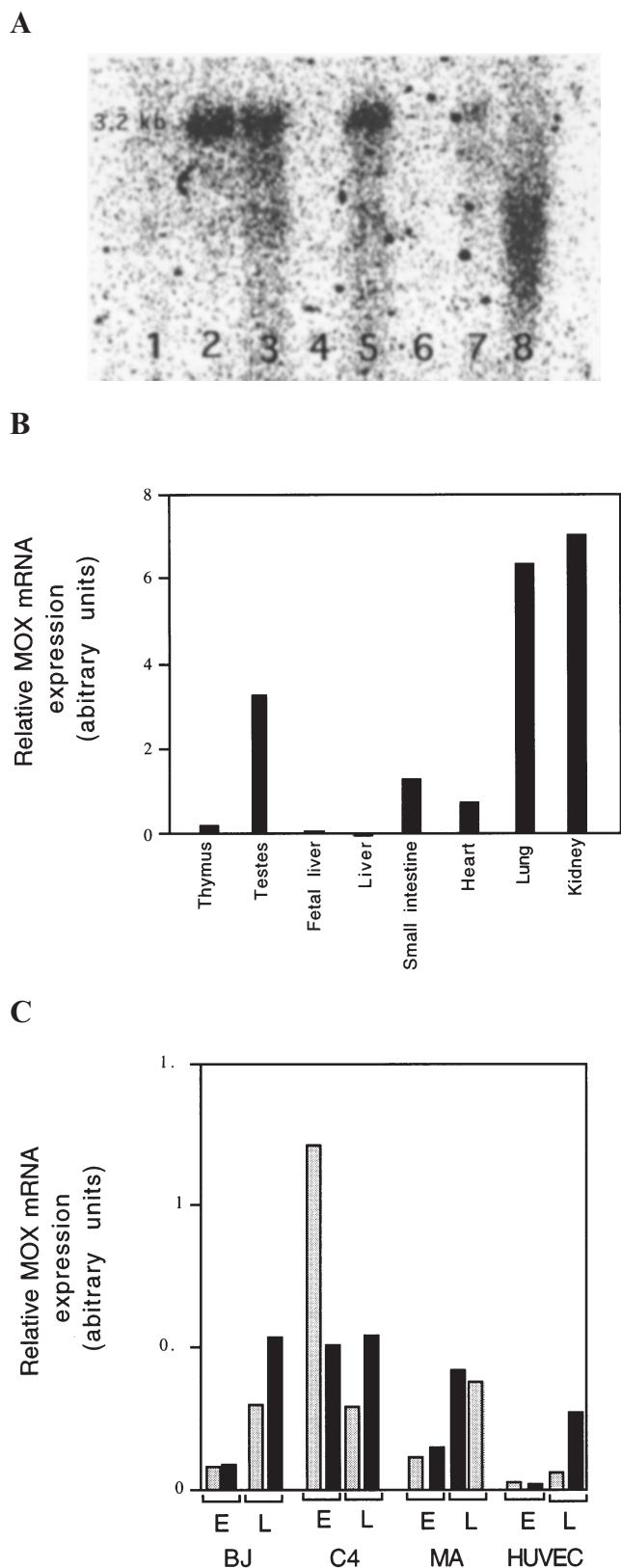


Fig. 3. Expression of MOX mRNA in human cells and tissues. (A) Northern blot analysis of human tissues. A Northern blot of total RNA (Invitrogen) from human tissues was probed with ^{32}P -labeled MOX probe (Section 2.3). Lane 1: heart; lane 2: brain; lane 3: kidney; lane 4: liver; lane 5: lung; lane 6: pancreas; lane 7: spleen; lane 8:

that DBH expression is restricted to adrenergic and noradrenergic cells of the adrenal gland, and the central and peripheral nervous system. EST expression data from the UniGene set (<http://www.ncbi.nlm.nih.gov/Schuler/UniGene/Hs.Home.html>) confirm that DBH is expressed predominantly in adrenal tissues.

To determine the expression of MOX mRNA in senescent human cell strains, RT-PCR was performed on total RNA samples. As seen in Fig. 3C, MOX mRNA levels were significantly higher in senescent BJ foreskin fibroblasts compared with early passage cultures, similar to the pattern we had observed previously for the original genetag GS6 (Linskens et al., 1995). This up-regulation was most pronounced in low serum conditions (0.5% FBS). A similar pattern of expression was also seen in MA dermal fibroblasts. However, in C4 dermal fibroblasts expression levels were highest in early passage cultures in high serum. In HUVEC cells, a vascular endothelial cell type, expression patterns were similar to those seen in BJ fibroblasts, although absolute expression levels were significantly lower than in fibroblast strains. Thus, changes in the steady-state levels of MOX mRNA are associated with replicative senescence in at least two cell types, and in two strains of the same cell type (BJ and MA skin fibroblasts).

3.4. Protein expression levels

A rabbit polyclonal antisera was raised against a glutathione S-transferase–MOX fusion protein and was used to assess MOX protein levels in human cell lines. As seen in Fig. 4, the antisera specifically recognizes a protein of approximately the predicted mobility of MOX (62.6 kDa). The expression levels appeared high in many of the cell lines tested. Using a partially purified source of bovine DBH, we were unable to detect cross-reactivity using the anti-MOX antisera, thus confirming the specificity of the reagent (data not shown).

3.5. Chromosomal localization

A radiation-hybrid panel was used to map the chromosomal localization of the MOX gene. MOX-specific primers were initially tested to identify pairs that could

skeletal muscle. (B) RT-PCR analysis of human tissues. Total RNA from human tissues as the starting material (Clontech). RT-PCR analysis was performed on total RNA (Clontech), using cDNA produced with random hexamer-primed total RNA. Expression levels for MOX are normalized to the signal from the 28S reactions (Section 2.4). (C) RT-PCR analysis of senescent human cells. RT-PCR was performed as for (B). MOX signals are reported as relative expression levels from early passage cells (E) or late passage cells (L). Human fibroblast strains: BJ, C4 and MA; human umbilical vein endothelial cells: HUVEC; □, high serum conditions; ■, low serum conditions.

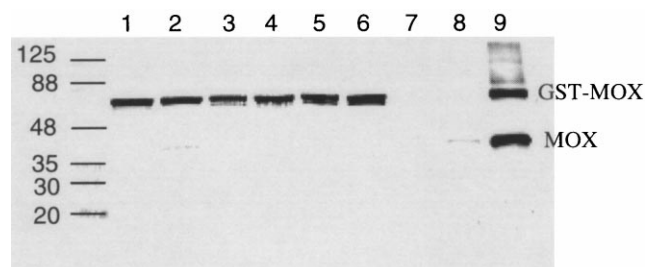


Fig. 4. Western blot analysis of MOX. Protein extracts (10 μ g total) from various human cell lines were resolved by SDS-PAGE, transferred to membranes, then probed with purified anti-MOX antisera. A preparation of Factor Xa-cleaved GST-MOX was included at various concentrations. Lane 1: adenovirus transformed embryonal kidney cells (HEK 293); lane 2: renal adenocarcinoma (ACHN); lane 3: epidermoid carcinoma (A-431); lane 4: breast adenocarcinoma (MCF7); lane 5: breast adenocarcinoma (MDA); lane 6: squamous cell carcinoma (Sc61-S9); lanes 7, 8, 9: factor Xa-cleaved GST-MOX at approximately 10, 100 and 1000 pg, respectively. Uncleaved GST-MOX (61 kDa) and cleaved partial polypeptide MOX (24 kDa) are indicated.

amplify an appropriately sized fragment from human genomic DNA, thus indicating that the primer pair interval did not span an intervening sequence. Analysis of the G3 radiation hybrid panel indicates that MOX localizes to chromosome 6q, with a lod score of 10.8 for linkage to marker D6S413 (Fig. 5). We are presently unaware of human genetic diseases that map tightly to this locus.

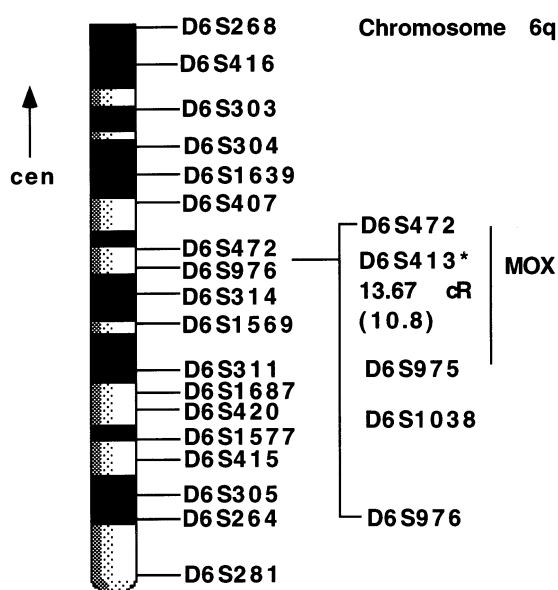


Fig. 5. Chromosomal localization. The chromosomal location of MOX was determined by using radiation hybrid panel mapping (Section 2.5). The closest marker was determined to be D6S413, on the long arm of chromosome 6. The estimated distance from D6S413 to MOX is indicated (1 cR = 26 kb) with the lod score in parentheses.

4. Discussion

We have identified and performed an initial characterization of a homolog of dopamine β -hydroxylase. Our results indicate that the steady-state mRNA levels of MOX are upregulated in some strains of cultured normal human cells at replicative senescence and that the expression pattern in human tissue partially overlaps with the patterns reported for DBH.

4.1. Homology with other monooxygenases

The unexpected identification of a clear homolog of DBH is interesting in view of the large number of studies that have investigated DBH biochemistry and function. Clearly, MOX can be assigned monooxygenase function due to the absolute retention of peptidyl copper ligands, structural features such as Cys residues and overall sequence identity with both DBH and PAM. However, the substrate specificity of MOX will be an important issue to resolve. DBH and PAM have relatively broad but distinct substrate specificities, with DBH preferring phenylethylamines, while PAM acts on glycol extended prohormones. The co-crystal structure of the monooxygenase domain of PAM bound with a substrate peptide shows that the protein interacts with substrate primarily through hydrogen bonds to the substrate peptide backbone. This relatively weak interaction may provide a structural basis for the wide substrate compatibility of the enzyme (Prigge et al., 1997). The conservation of at least one substrate-binding residue in MOX, PAM and DBH (Y400 in hMOX, Y477, hDBH and Y318 in rat PAM) suggests similarity in binding at the active sites.

We have performed some initial experiments to express recombinant forms of MOX in eukaryotic cells for the purpose of testing for catalytic activity. In vitro transcription-translation of the MOX cDNA provides a protein of the predicted mobility that was inactive in a DBH activity assay (data not shown) (Li et al., 1996). Expression of active recombinant DBH enzyme has been reported (Lewis and Asnani, 1992; Gibson et al., 1993, Li et al., 1996) and may prove instructive in the expression of MOX for further analysis of the catalytic activity. Alternatively, the antisera generated in this study could be used to purify the native enzyme from cell or tissue sources.

The absence of an obvious signal sequence for MOX suggests that the protein may be retained intracellularly. The signal sequence of DBH has been the focus of numerous investigations. Uncleaved versions of DBH that retain their signal peptides have been identified (Robertson et al., 1990; Wang et al., 1990) and suggestions have been advanced that the association of the protein with membranes is mediated through the remaining signal peptide (Taljanidisz et al., 1989). Other studies have shown that recombinant versions of DBH that

lack a signal peptide are still capable of associating with membranes (Lewis and Asnani, 1992; Gibson et al., 1993), and thus in a similar manner, MOX may also associate with membranes, even in the absence of a well-defined signal peptide sequence. We note a region of predicted hydrophobicity at the extreme C-terminus of the MOX protein (Fig. 1C) that may facilitate interactions with membranes.

4.2. mRNA expression patterns

The MOX mRNA is expressed in a senescence-associated manner in a subset of fibroblast strains, but in others expression is not increased by replicative senescence. These results, and those of many other studies, indicate that there is considerable variability in gene expression patterns at senescence, depending on the cell type and strain under examination (Linskens et al., 1995; Takeda et al., 1992). The signalling pathway(s) activated at senescence result in an irretrievable cell cycle arrest, an event common to almost all normal diploid human cell types (Campisi, 1997). In concert with this arrest, a large-scale alteration of gene expression is affected, that may include a large degree of collateral gene regulation that will vary depending on the differentiation status of the cell (Cristafolo and Pignolo, 1996). Using high density DNA array technologies, we have recently observed a similar up-regulation of PAM mRNA expression in senescent BJ fibroblasts (W.F. D.S. and E.C. unpublished observations). Whether or not senescence-associated regulation is a common feature of other monooxygenases remains to be determined. It also will be of interest to examine whether the activation of MOX follows that of DBH, which is known to be activated by cAMP, glucocorticoids, calcium, and growth factors (Lewis et al., 1987; Stachowiak et al., 1990a; Stachowiak et al., 1990b; Kim et al., 1993; Acheson et al., 1994).

MOX mRNA expression patterns appear to be less restricted than that of DBH, with high levels of expression seen in the lung, kidney and adrenal gland. It appears unlikely that MOX contributes significantly to norepinephrine production, since DBH knockout mice have undetectable levels of norepinephrine in their tissues or in serum (Thomas et al., 1995; Thomas and Palmiter, 1997). Whether MOX mRNA shares a pattern of expression with DBH in noradrenergic or adrenergic neurons will be of particular interest.

The results presented here should allow for a detailed biochemical analysis of the MOX gene and protein. A direct demonstration of the substrate specificity of this monooxygenase would substantially define the biochemical circuitry of this enzyme. In the absence of any candidate diseases tightly associated with the chromosomal locus for the MOX gene, a knockout phenotype

in either cells or mice may be instructive in defining additional biological roles for the enzyme.

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